RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Development of methods for determining the estrogen content of swine wastes: **NPB 04-204**

Investigator: Charles Scott Whisnant

Institution: North Carolina State University

Date Received: July 14, 2006

Abstract
This project was designed to compare methods for measuring estrogen levels in swine waste from different types of production facilities and the effects of holding the waste on the concentrations of different forms of estrogen. We validated extraction methods for urine, feces and lagoon water and used the extracted material in radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA) and gas chromatography-mass spectrometry (GC-MS) to measure the estrogen concentrations. All three methods can be used to measure estrogens but RIA and GC-MS were more repeatable in our study than the ELISA.

Comparison of different types of production facilities indicated that while the boar stud and the sow breeding and gestation units had the highest level of estrogens the finisher units also had significant levels of estrogens in waste material, which fits with what we know of hormone production by pigs. No significant seasonal differences were found but winter levels tended to be higher. Holding of the waste resulted in decreased levels of the more potent estrogens presumably due to their conversion to less potent forms by bacterial activity.

Introduction
Estrogens are natural products of the endocrine system of most animals and therefore will be present in the wastes excreted by livestock including pigs. Limited information exists concerning the level of estrogens in swine waste. Studies conducted with livestock waste have used a variety of methods for extracting and measuring the steroid compounds. This has resulted in sometimes conflicting data. The current proposal was designed to test and compare different methods of measuring estrogens in order to help develop standard methods that could be used across the industry and to determine the level of estrogens present in the waste of different types of pork production facilities.

Objectives
1. Review the scientific literature on estrogens in the waste stream and the environmental fate of estrogens from swine facilities. Results from the literature will be compared with those obtained from the experiments conducted as part of the objectives listed below.
2. To develop and validate standardized methods for determining the concentration and biological activity of estrogenic compounds in swine urine, feces, and lagoon wastewater. Results from radioimunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) will be compared with those from gas chromatography-mass spectrometry (GC-MS).
3. Use these methods to determine concentrations of estrogenic compounds in production facilities expected to

differ in content due to different stages of production and types of pigs housed in the facilities.
4. Use the yeast estrogen screen bioassay to determine the bioactivity of estrogenic compounds in swine waste and compare the bioactivity with the immunoactivity and specific concentrations determined by methods of objective 2.
5. Use the methods validated in objective 2 to measure the effect of environmental factors on estrogenic concentrations in swine waste stored in various holding facilities.

Materials and Methods

The review of the literature made it evident that several methods had been used to measure estrogen content in wastewater from swine or other animals, including humans. In order to develop and validate standardized methods it was decided to collect wastewater from the lagoon of the North Carolina State University Swine Educational Unit (SEU) and to compare different extraction methods to purify the samples and then to take those samples and measure them using RIA, ELISA and GC-MS. In addition to wastewater samples, feces and urine samples were collected from pregnant sows at the SEU to compare estrogen concentrations in fresh excreta with that from the lagoon. It was decided to collect samples from sows for ease of collection and because they should have high estrogen concentrations.

Extraction Methods

Fecal

0.5 g of feces was mixed with 4.5 ml of 90% methanol by shaking for 40 minutes. The mix was then centrifuged at 1500 x g for 15 minutes. The supernatant was then evaporated under pure nitrogen gas and reconstituted in estrogen free serum, produced by charcoal stripping of a porcine serum pool.

Urine

500 ul of urine samples were extracted with 1.0 ml dichloromethane by mixing with gentle inversion for 5 minutes. The mix was centrifuged at 1500 x g for 5 minutes. The upper phase was removed by aspiration. 50 ul of the lower phase was used directly in the assay.

Lagoon water

The extraction began with adding 2.7 ml of 37% formaldehyde to 100 ml of lagoon water. Addition of formaldehyde has been shown to be one method of preventing bacterial degradation of estrogens. This mixture was stirred for 5 minutes while CO_2 was vented. The liquid was centrifuged to precipitate solids and the liquid was then filtered 50g of glass filter aid located on top of a 90 mm APFC filter. The filtered fluid was then put through solid phase extraction using Oasis® HLB cartridges from Waters corporation () using the following protocol. The cartridge was conditioned by running 3 ml of diethyl ether through it, then rinsed with 3 ml of methanol and 3 ml of distilled water. After running of the sample the column was washed with 3 ml of 5% methanol in distilled water and eluted with 6 ml of 10% methanol in diethyl ether. The liquid was dried down under a gentle nitrogen stream and the reconstituted with assay buffer from one of the commercial kits for RIA and ELISA. For GC-MS the sample was derivatized with 450 ul of dimethylformamide and 50 ul of BSTFA (N,O-bis, trimethylsilyl, trifluoroacetamide).

Assay methods

RIA Procedure

The estradiol assay used was a double antibody assay from Diagnostic Products Corporation (Los Angeles, CA) and the estrone assay was from Diagnostic Systems Laboratories (Webster, TX) and used according to manufacturer instructions.

ELISA Procedure

Kits were purchased from Cayman Chemical (Ann Arbor, MI) for E2, E1, and E1 sulfate and used according to manufacturer instructions.

GC-MS Procedure

Derivatized samples were analyzed on a HP 6890 gas chromatograph with a DB-5MS capillary column using a HP 5973 mass selective detector. The standard curves consisted of 5 points from one to 5,000 ppb and were made using purified estrogens purchased from Sigma Chemical (St. Louis, MO). The primary ions for quantification were m/z 416 for estradiol and m/z 342 for estrone.

Results

Review of the Literature

Livestock produce and excrete hormones as part of the physiological processes needed to maintain themselves, grow and reproduce. Large numbers of livestock found in CAFOs could represent significant sources of these excreted hormones. Concerns have been raised about the environmental impact of these hormones, especially estrogens and androgens, because research has shown that concentrations in the low parts-per-trillion range can affect the reproduction of aquatic wildlife (Tyler et al., 1998; Orlando et al., 2004) by acting as endocrine disrupters. Much of the endocrine disruption literature has dealt with pollution containing synthetic estrogens or human waste, which typically is high in estrogens due to their use in contraceptives and other medications. However endocrine disrupting effects have been demonstrated in fish exposed to effluent from a cattle feedlot (Orlando et al., 2004). Research in this area has concentrated on estrogens and their effects. Irwin et al., (2001) found that turtles living in ponds receiving runoff from pastures fertilized with cattle manure had higher levels of vitellogenin than turtles from nearby control ponds indicating that they were responding to estrogens. Peterson et al., (2000) used estrogen levels in water from an aquifer as indicator of livestock waste application. Human health risks from estrogen exposure have not been established but some groups have raised concerns there as well.

Although it is the most potent naturally occurring estrogen, 17-β estradiol (E2β) may not have the most environmental impact because of its conversion to estrone (E1) or other estrogens. Fine et al (2003) in their study to validate a method for measuring estrogens in swine lagoon samples found it was necessary to kill the microbes present in the sample because otherwise all the added E2β was converted to E1. Hanselman and colleagues (2003) reported that while E2β and estradiol 17-α (E2α) could be detected in the pit below a farrowing house, only E1 could be detected in the lagoon for the farrowing house. This indicates that the estradiol forms are converted to E1 with time. Estrone was also the only detectable estrogen in a lagoon containing waste from a finishing house. The concentration of E1 in the finishing house lagoon was significantly lower than in the lagoon from the sow unit as would be expected (Hanselman et al., 2003).

The pork industry largely utilizes multi-site production systems. For example a group of sow barns would be located together but separate from nursery, grower-finisher or boar units, which would be separate from each other as well. This will cause different units to have different levels and types of hormones present in their waste. For example only boar studs would be expected to have significant concentrations of androgens but boars also have high blood levels of estradiol (At-Taras et al., 2006). However, very few boar studs exist because artificial insemination allows one boar to breed many sows with each ejaculate, and most boar studs are too small to be considered a CAFO. Sow farms should have higher progesterone concentrations than finishers because most sows will be pregnant. Finishing barns housing only barrows would not be expected to contain significant amounts of steroids in the stored manure. Manure from mixed sex finishers may contain higher levels of steroid hormones, but this is uncertain since most gilts will be prepubertal until near the end of their finishing period.

Very little work has been done measuring progestagens in the environment or in their effects on wildlife. Lorenzen et al., (2004) using *in vitro* receptor assays for estrogens, androgens and progesterone activity surveyed levels in livestock and human wastes. No progesterone receptor activity was reported for any swine or cattle sample although activity was detected in one poultry litter sample. However samples from cattle fed melengestrol acetate (MGA), a synthetic progestagen had no receptor activity in the Lorenzen study. This negative result may indicate that the receptor assay may not work as well for progesterone receptors as it did for the other steroid receptors or the progestagen compounds may be broken down more quickly and thoroughly. Further research is needed with progestagens. Lorenzen and coworkers (2004) reported low levels of androgens in lagoons from sow farms but nothing was detectable from finishers or nurseries. The authors did not sample boar farms or state if boars were housed on the sow farms.

Most livestock manure is applied to fields for crop production or pastures used for grazing. Runoff from these fields could potentially affect wildlife. Indeed changes in fish have been reported (Ankley et al., 2003; Orlando et al., 2004) in streams receiving runoff from cattle feedlots. Shore et al., (1993) reported that testosterone was readily washed from soil by aqueous solutions (>90%) but estrogens were more likely to be retained (~50%) in the soil after the same treatment. Estradiol 17-β is quickly converted to the less potent E1 in agricultural soils (Colucci and Topp, 2002) but the fate of the E1 differed according to soil type. Estrone persisted much longer in silt loam and sandy loam soils than in loam. The conversion of E2β to E1 was not affected by autoclaving the soil, which either means the soil was not completely sterilized or the conversion can take place by abiotic mechanisms. Estrone was stable in autoclaved soils. Several studies have found that E1 is degraded by common soil microorganisms (Hanselman et al., 2003). Colucci et al., (2001) found that E2 and E1 quickly formed non-extractable residues in non-sterile soils. Sorption of E2 and E1 equilibrated within 5-24 hours (Casey et al., 2005). Lorenzen et al., (2005) looked at degradation of 3^{H}- testosterone in three soil types and found that it was quickly degraded with half lives ranging from 8.5 to 21 hours. They concluded that it was rapidly degraded and unlikely to be of ecological concern. Casey et al., (2004) also found that testosterone was more quickly degraded that E2 but that it was more likely to move through the soil due to lower sorption. No data in the literature were found for progesterone. The types of soils found in large pork producing areas need to be examined.

Ultimately it is the biological activity of estrogens in waste and runoff that causes environmental concerns. Therefore the estrogenic activity present in waste samples needs to be assessed by some type of bioassay. The bioassay methods have the advantage of measuring the estrogenic activity of all estrogens present in the sample whereas analytical methods for determining concentrations of specific estrogens must be done for each metabolite. The two most commonly used methods are the E-SCREEN bioassay, based on proliferation of MCF-7 cells and the yeast estrogen screen (YES). Soto et al., (2004) used MCF-7 cells to determine the estrogenic activity of effluent from a cattle feedlot. The yeast estrogen screen uses yeast cells, which have been transfected with the estradiol receptor alpha (Routledge and Sumpter, 1996). The ER gene is linked to a reporter gene, beta- galactosidase that can be detected and increases over control of the expression of the reporter used to determine the response to estrogen (Burnison et al., 2003). In the only study found to have examined estrogen bioactivity in hog waste, Burnison et al., (2003) reported that the majority of the activity in lagoon waste from a sow farm came from E1 and E2β. This indicates that detection of E1 and E2β may be sufficient to characterize estrogens in manure samples. Burnison et al., (2003) also collected tile drain water after a rain event from a field to which the sow lagoon waste had been had been applied and detected modest levels of estrogenic bioactivity in the runoff. A similar assay has been used to measure androgen activity in runoff from cattle feedlots (Orlando et al., 2004). As mentioned above very little has been done with progestagen compounds, which were detected by an in vitro receptor assay in only one sample (Lorenzen et al., 2004). More detailed characterization of steroid levels in swine wastes and their fate after land application are needed.

Environmental Data Collection

Samples were collected from ten sites in each of the four seasons to compare estrogen content from different types of production facilities and the effect of season on estrogen content in the lagoon from these facilities. The review of the literature (objective 1) was done as part of the application process and the investigators have continued to review literature as the research was conducted. Results from the current research will be compared with published results in the discussion below.

Comparison of estrogen concentrations from the three methods revealed that although actual concentrations as measured by the methods differed, a similar ranking of the sites was found with all three methods. Ranking from highest to lowest concentrations is given below. Concentrations are in ng per Liter.

Rank	Site
1	Boar
2	Sow-B
3	Sow-D1
4	Sow-D2
5	Sow-S2
6	Sow-S1
7	Finisher-H
8	Finisher-S
9	Finisher-B
10	Nursery

Table 1. Ranking of pork production sites from highest to lowest in concentrations of estrogens found in lagoon wastewater.

Summer	E2	E2	E2	E1	E1	E1
Site	RIA	ELISA	GC-MS	RIA	ELISA	GC-MS
Boar	8257	9116	7487	18,550	21,789	15,575
Sow-B	7763	8142	7395	14,359	16,974	13,025
Sow-D1	7046	7559	6863	12,674	13,873	10,985
Sow-D2	6947	7369	6147	10,411	11,672	9873
Sow-S2	5485	5967	5139	9876	10,754	8462
Sow-S1	4933	5144	4275	9248	10,367	8138
Finisher-H	2108	2347	1856	7142	7782	6835
Finisher-S	2044	2259	1841	7284	7651	6945
Finisher-B	1732	2007	1665	6350	6770	6290
Nursery	31	34	22	375	402	357

Autumn	E2	E2	E2	E1	E1	E1
Site	RIA	ELISA	GC-MS	RIA	ELISA	GC-MS
Boar	7945	8330	7060	16,663	18,796	14,995
Sow-B	7875	8220	7560	13,457	14,420	12,460
Sow-D1	6873	7127	6650	13,330	13,964	11,770
Sow-D2	5190	5465	4980	9754	10,967	9645

Sow-S2	4895	5055	4780	9643	10,457	9350
Sow-S1	5075	5235	4865	8954	9983	8520
Finisher-H	1798	2043	1725	7545	8247	7277
Finisher-S	1865	2122	1675	7378	8168	6975
Finisher-B	1650	1920	1490	6198	6557	5980
Nursery	45	55	30	390	425	365

Winter	E2	E2	E2	E1	E1	E1
Site	RIA	ELISA	GC-MS	RIA	ELISA	GC-MS
Boar	9367	10,104	8567	13,354	14,745	12,960
Sow-B	8643	9287	8025	12,260	14,122	10,890
Sow-D1	7390	7682	6953	11,478	12,770	9984
Sow-D2	6457	6689	5985	11,273	12,585	9960
Sow-S2	5981	6429	5675	10,474	11,912	9285
Sow-S1	5476	5873	5230	9890	10,244	8955
Finisher-H	2344	2556	2175	6884	7543	6529
Finisher-S	2076	2148	1835	6672	7346	6320
Finisher-B	2194	2488	1965	5490	5570	5280
Nursery	65	77	58	485	504	454

Table 2. Concentrations of estradiol (E2) and estrone (E1) from pork production lagoons as measured by radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), and gas chromatography-mass spectrometry (GC-MS) during three seasons.

Time	E2	E1	E2 Glucuronide	E1 Sulfate
0 h	4846	7187	1152	6342
24 h	679	10,876	ND	5106
48 h	459	9734	ND	3955
96 h	383	9442	ND	2661

Table 3. Effects of time of storage on concentrations of estradiol (E2), estrone (E1), estradiol glucuronide (E2 glucuronide) and estrone sulfate (E1 sulfate) as measured by enzyme-linked immunosorbent assay (ELISA).

Discussion

The methods evaluated in the current project gave a similar ranking to the sites sampled and across seasons, which likely indicates that the values reflect the actual concentrations. Each method has advantages and disadvantages. The GC-MS requires substantial cleanup and solid phase extraction but does allow measurement of all compounds of interest at the same time. The immunoassay methods may as others have pointed out overestimate steroid levels since there is some cross-reactivity of the hormones with the antibodies used. For example the antibody used in the E2 ELISA had a cross-reactivity of 17% estradiol glucuronide and 4% with E1 and the antibody used in the E1 ELISA had a 100% cross reactivity with estrone sulfate. Each

compound of interest must be assayed separately with the immunoassay techniques. However the RIA has been reported to be more sensitive and involve less complicated methods of extraction. Any of the methods can provide useful information however the GC-MS or other chromatographic methods are more likely to be amenable to standardization.

Comparing estrogen concentrations between sites reveals that the highest concentrations were found in the lagoon from the boar stud, with concentrations decreasing from sow farms to finishers to nursery sites. Only one boar stud was sampled and it is possible that the high readings could be due to some other factor but it is known that boars produce much higher levels of estrogens (Louis et al., 1994; At-Taras et al., 2006) than found in males of other species. Lange et al., (2002) used data from published reports of estrogen levels in freshly collected feces and urine to calculate yearly steroid production by various types of farm animals and concluded that boars would excrete more estrogens than sows. This has not been tested experimentally but seems reasonable and would fit with the current data. No reports in the literature of concentrations from boar stud lagoons were found. Finding higher estrogen levels in lagoons from sows than from finisher and nursery lagoons was expected and fits with the limited data in the literature (Williams, 2002; Fine et al., 2003). Finisher units sampled were gilts only or mixed with barrows and gilts. Units with only barrows would not be expected to produce measurable estrogen concentrations.

Season
Winter E2 levels (data shown for E2 and E1 in tables above) were higher than in summer or fall. Levels of E1 were not different. This could reflect lower bacterial activity due to colder temperatures, which would decrease conversion of E2 to E1 or differences in precipitation, could have affected this.

Storage
Estradiol concentrations decreased with time stored. Estrone concentrations did not decrease and actually increased presumably due to conversion of E2 to E1. Some of the conjugated forms were reduced by storage time Estrogen glucoronides were not detectable by 24 hours after storage. Estrone sulfate was detectable at 96 hours after storage although it was reduced by approximately 60% from time 0.

Lay Interpretation

Measurable levels of estrogens were found in lagoons from all types of production with boars and sows being higher than finishing units. The number of nursery units sampled was limited but those still had levels that could affect fish based on toxicology studies. Levels were below what has been reported for human sewage and cattle feedlots. Storage reduced levels of the most potent estrogens. Runoff from fields was not been tested but based on the limited published research, rainfall events shortly after land application (Burnison et al., 2003) could result in detectable estrogen levels in groundwater. Methods of storage and different types of ground application need to be tested. Storage under lab conditions reduced the estradiol concentration. Since most estrogens are excreted from pigs in urine systems that separate urine and feces may be of use in reducing estrogens in the environment from pork production.

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